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Mega Base Map of the Epidermal Growth Factor (EGF) Receptor Gene Flanking Regions and Structure of the Amplification Units in EGF Receptor-hyperproducing Squamous Carcinoma Cells

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We have established a mega base scale physical map of the 5′- and 3′-flanking regions of the epidermal growth factor (EGF) receptor gene using CpG-recognition rare-cutting restriction enzymes and pulsed-field gel electrophoresis. In this map, a methylation-free CpG island (HTF island) is located within an 8-kilobase pair (kb) *Eco*RI fragment which includes exon 1 of the EGF receptor gene. From this HTF island, a 390-kb *Not*I fragment was identified as the longest 5′-flanking region and a 540-kb *Mlu*I fragment as the longest 3′-flanking region. Utilizing this map information, we have analyzed the structure of the flanking regions of amplified EGF receptor genes which are found in various squamous carcinoma cells. Among seven cell lines tested, four cell lines carrying EGF receptor genes in amounts more than 20 times that of normal cells showed amplification together with large 5′- and 3′- flanking regions. The amplified 5′-flanking regions were rearranged in different forms but were distinct in each cell line. The amplified 3′-flanking regions were at least 540 kb in size and common to all the cell lines, except that A431 had rearrangement points within 540 kb downstream of the HTF island. Thus, the size of amplification units appears to be large and different in each cell line.

Key words: EGF receptor — Gene amplification — Squamous cell carcinoma — Pulsed-field gel electrophoresis — Restriction map

DNA sequence amplification has been described in a number of drug-resistant cells and cancerous cells and tissues.[1-3] Amplification of the EGF[*4] receptor gene is often seen in gliomas[4] and squamous cell carcinomas.[5] This amplification appears to be uncorrelated with such chromosomal abnormalities as homogeneously staining region (HSR) or double minute chromosomes (DMs) which are often seen in association with N-*myc* oncogene amplification.[6]

Recently, it has been demonstrated that inverted duplications are involved in amplification of a polyoma DNA segment,[7] and c-*myc* and CAD genes,[8] and that both inverted and direct repeats are found in dihydrofolate reductase (DHFR) genes in methotrexate-resistant cells.[9] Although the nature of amplified DNA sequences has been described and some hypotheses have been presented,[3, 10, 11] the mechanism of gene amplification is not yet completely understood.

In this paper, we describe the physical map of EGF receptor gene flanking regions over 1 megabase pair (Mb) in size, and the structure of these regions in several human squamous carcinoma cell lines in which amplification of the EGF receptor gene had occurred.

MATERIALS AND METHODS

Cell Lines and Cultures Human lymphoblast cell line GM 130 B was maintained in RPMI 1640 supplemented with 15% FCS. Other human carcinoma cell lines and somatic cell hybrids were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Characteristics of the eight cell lines used in this experiment are summarized in Table I. Human epidermoid carcinoma cell line A431[12] was kindly provided by Dr. S. Cohen (Vanderbilt University), NA, Ca9-22, HSC2, and HSC3 by Dr. K. Rikimaru (Tokyo Medical and Dental University),[13, 14] and TE1 and TE8[13] by Dr. T. Nishihara (Tohoku University).

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[*4] Abbreviations: EGF, epidermal growth factor; kb, kilobase pair; FCS, fetal calf serum; SDS, sodium dodecyl sulfate.

GM130B cells were obtained through the Camden Cell Bank.[15]

DNA Preparations and Southern Blot Analysis High-molecular-weight DNA was purified from various cell lines and placenta by the SDS-proteinase K cell lysis method, followed by phenol-chloroform extraction, and ethanol precipitation. DNA was digested with *Eco*RI, followed by ethanol precipitation. Some DNA samples were further digested with appropriate enzymes, separated by agarose gel electrophoresis and transferred to Gene Screen Plus (New England Nuclear) by the method of Southern.[16] Prehybridization and hybridization were performed according to the commercial suppliers' instructions. The final wash was for 60 min at 55° in 0.5×SSC (1×SSC: 0.15*M* NaCl, 15m*M* sodium citrate)−1% SDS. Autoradiography was at −80° on Fuji New RX film with a DuPont Cronex Lightning-Plus screen.

Orthogonal-Field-Alternation Gel Electrophoresis (OFAGE) Samples were further prepared as described by Gardiner *et al.*[17] DNA was digested with 40 U of restriction enzyme for each sample after soaking in 500 μl of reaction buffer for 2 hr at 4°. OFAGE[18] was carried out using a Pulsaphor system (LKB). Electrophoresis conditions were 1% agarose, 10 V/cm voltage gradient, non-homogeneous field of 330 V alternating every 45 sec, 0.05*M* TBE (1*M* TBE: 1*M* Tris, 1*M* boric acid, 20m*M* EDTA) cooled to 8°, and a 35 hr electrophoresis time. After DNA separation by OFAGE, Southern transfer, hybridization and washings were performed as described above. The same filter was sequentially used for rehybridization.

DNA Probes EGF receptor cDNAs pE15, pE7 and pE62 were kindly provided by Merlino *et al.*[19] A 5′-end *Cla*I-*Eco*RI 450-bp fragment of pE15 insert [probe C], 2.4-kb pE7 insert [probe D] and 1.08-kb pE62 insert [probe E] were used as probes for Southern hybridization or cloning procedures. An *Eco*RI 8-kb fragment from human placental DNA which hybridizes to probe C and contains exon 1 of the EGF receptor gene was cloned into λgtWES•λB after fractionation by agarose gel electrophoresis. The insert was subsequently recloned into pUC18 [pERP8]. A 5′-end *Eco*RI-*Hin*dIII 2.2-kb fragment [probe A], and a 3′-end *Pvu*II-*Eco*RI 400-bp fragment [probe B] were used as probes for Southern hybridization. These probes were labeled with ^{32}P by a random priming method.[20]

Results

HTF Island in EGF Receptor Gene Promoter Region We have identified an 8-kb fragment corresponding to exon 1 of the EGF receptor gene[21] in *Eco*RI digests of human lymphoblastoid line GM130B DNA using probe C (5′-end of pE15). This 8-kb fragment was also detected in human placental DNA and subsequently cloned into a plasmid vector. From the resulting pERP8 clone, a 5′-end 2.2-kb *Eco*RI-*Hin*dIII fragment and a 3′-end 0.4-kb *Pvu*II-*Eco*RI fragment were cut out for use as probes (A and B) in further analysis.

DNA sequence data indicate that the promoter region of the EGF receptor gene is extremely rich in G+C content and the relatively rare CpG configuration occurs frequently.[21] We therefore examined whether the 8-kb DNA fragment is susceptible to digestion by restriction enzymes *Sac*II, *Mlu*I and *Not*I, which preferentially cut at methylation-free CpG-containing sequences. These restriction enzymes are known to cut the mammalian genome infrequently,[22] and the sites available for cleavage by these rare-cutting enzymes tend to occur in clusters which correspond to regions rich in non-methylated CpG.[23] Genomic DNA from GM-130B was cut with *Eco*RI followed by these enzymes. As shown in Fig. 1, these enzymes cut the 8-kb *Eco*RI fragment into smaller segments of which one segment each was detected by probe A. Further analysis using

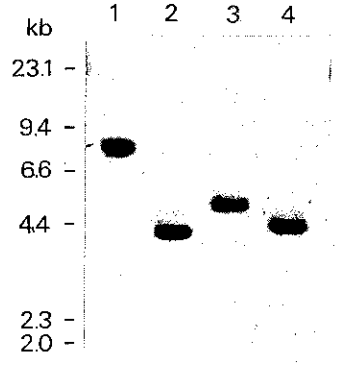


Fig. 1. Southern hybridization analysis of the 8-kb *Eco*RI fragment from GM130B. Genomic DNA (2 μg) purified from GM130B was digested with *Eco*RI, followed by second digestion with one of the rare-cutting enzymes, *Sac*II (lane 2), *Mlu*I (lane 3), or *Not*I (lane 4). Southern hybridization was performed using probe A. Lane 1 was loaded with *Eco*RI digest to show 8-kb fragment. *Hin*dIII-digested λDNA was used as a size marker.

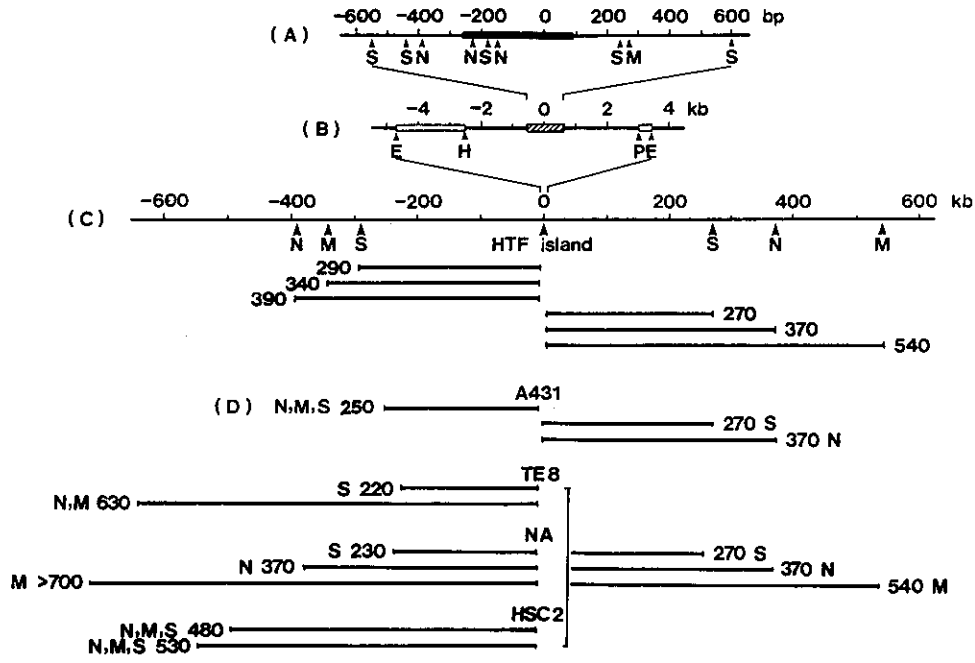


Fig. 2. Physical map of the EGF receptor gene promoter regions and flanking regions. (A) Restriction map of the HTF island. The closed box represents exon 1 of the EGF receptor gene. (B) Restriction map of the pERP8 insert. The open box represent the location of probe A and probe B, and the shaded box represents the HTF island. (C) Physical map of the 5′- and 3′-flanking regions of EGF receptor gene in GM130B cells. Horizontal lines represent the length and location of restriction fragments detected in Fig. 3. (D) Size and location of restriction fragments seen in squamous cell carcinoma lines. Restriction enzymes used were *Sac*II (S), *Mlu*I (M), *Not*I (N), *Eco*RI (E), *Hin*dIII (H) and *Pvu*II (P). Each triangle represents a recognition site of a restriction enzyme. Sizes described in kb.

these methylation-sensitive rare-cutting enzymes enabled us to construct a restriction map of this 8-kb fragment of pERP8 (Fig. 2 A, B). The restriction map indicates the presence of a methylation-free CpG island, also known as an *Hpa*II tiny fragment (HTF) island,[24] in the center of the 8-kb fragment.

EGF Receptor Gene Flanking Regions GM-130B DNA was digested with three rare-cutting restriction enzymes and fragments were separated by pulsed-field gel electrophoresis. Southern blot analysis using probe A (5′-end of the 8-kb segment) revealed fragments of 340, 290 and 390 kb in digests from *Mlu*I, *Sac*II and *Not*I, respectively (Fig. 3A). This probe also detected a weak *Mlu*I-fragment smaller than 260 kb (Fig. 3A, lane 1). This may be a partial digestion product caused by partial methylation at this *Mlu*I site. These fragments correspond to the flanking region upstream of the HTF island. Similar analysis using probe B (3′-end of the 8-kb segment) revealed fragments of 540, 270 and 370 kb in these digests, respectively (Fig. 3B). Analysis using EGF receptor cDNA probes, probe D (pE7) and probe E (pE62), provided the same restriction patterns (data not shown). These fragments must include the entire EGF receptor structural gene and its flanking regions because the genomic size of the EGF receptor gene has been estimated to be about 110 kb.[25] Although these three enzymes are sensitive to methylation, this did not disturb analysis of these data. We always observed one or two major bands in each lane. These results enabled us to construct a restriction

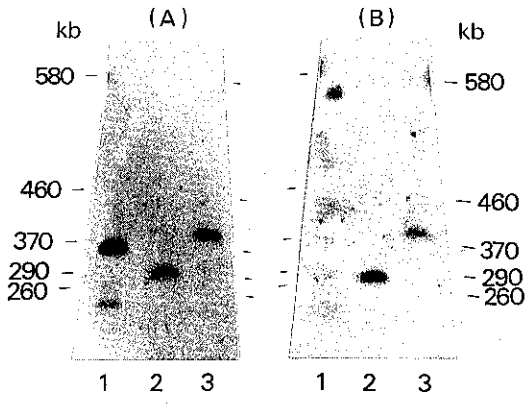


Fig. 3. Southern hybridization analysis of flanking regions of the EGF receptor gene. DNA of GM130B was digested with *Mlu*I (lane 1), *Sac*II (lane 2), or *Not*I (lane 3), separated by OFAGE, and analyzed by Southern hybridization using probe A (A), or probe B (B). Yeast (X2180-1A) chromosomes were used as size markers and are indicated on both sides of the figures.

Table I. Amplification of the EGF Receptor Gene in Various Cell Lines

Cell line	Origin	Degree of EGF receptor amplification[a]
GM130B	B cell	×1
A431	Epidermoid carcinoma, vulva	×20
NA	SCC,* tongue	×30
Ca9-22	SCC, gingiva	×5
HSC2	SCC, mouth	×40
HSC3	SCC, tongue	×5
TE1	SCC, esophagus	×3
TE8	SCC, esophagus	×20

* SCC; squamous cell carcinoma.
a) The degree of amplification was described by Yamamoto *et al.*[13]

map of the 930-kb flanking regions of the EGF receptor gene (Fig. 2C).

5′-Flanking Regions of Amplified EGF Receptor Genes Table I lists human squamous carcinoma cells which carry amplified EGF receptor genes. DNAs from these tumor cells were digested with rare-cutting restriction enzymes and analyzed as above. Southern blot analysis using probe A revealed apparently rearranged fragments which were not seen in GM130B DNA (Fig. 4A). A431 cells showed a 250-kb fragment in each of the three digests (Fig. 4A-a). TE8 cells showed a 630-kb fragment in common, although *Sac*II fragment was detected in a minute amount (Fig. 4A-b). Three to four other fragments of 300 and 400 kb were also detected. NA cells showed fragments of 230 (*Sac*II), 370 (*Not*I) and more than 700 kb (*Mlu*I) in addition to a minute amount of normal-sized fragments detected in GM130B (Fig. 4A-c). HSC-2 cells showed fragments of two different sizes (480 and 530 kb) in common (Fig. 4A-d). In all these cases, hybridization intensity of major fragments apparently reflected most of the amplified EGF receptor genes. These results indicate that the EGF receptor gene is amplified together with a large 5′-flanking region and that the amplified units are discrete but differ among cell lines. Furthermore, the restriction fragments common to the three rare-cutting enzymes represents the presence of the same clusters in each of three cell lines (A431, HSC2 and TE8). These amplified fragments might be arranged in a similar manner, because *Not*I and *Mlu*I sites are generally very rare even in HTF islands.[22]

3′-Flanking Regions of Amplified EGF Receptor Genes Southern blot analysis of NA, TE8 and HSC2 DNAs using probe D (pE7) revealed three major fragments (270, 370 and 540 kb) (Fig. 4B-b, c, d) which are seen in GM130B (Fig. 3B). There are some minor fragments which may represent partial digests or minor rearrangement during the amplification process. A431 DNA, however, showed a different pattern when compared to the other three cell lines (Fig. 4B-a). A 370-kb *Not*I fragment which is observed in GM130B cells, is also present but there exist two major *Sac*II fragments: one is normal 270-kb fragment and the other is an about 180-kb fragment which may be produced by partial demethylation or rearrangement of 3′-flanking sequences. The total hybridization intensity of these two *Sac*II fragments is almost the same as that of 370-kb *Not*I fragment, which represents the greater part of the amplified EGF receptor genes in A431. On the other hand, there is no distinct amplified *Mlu*I fragment, indicating that the *Mlu*I fragments containing EGF re-

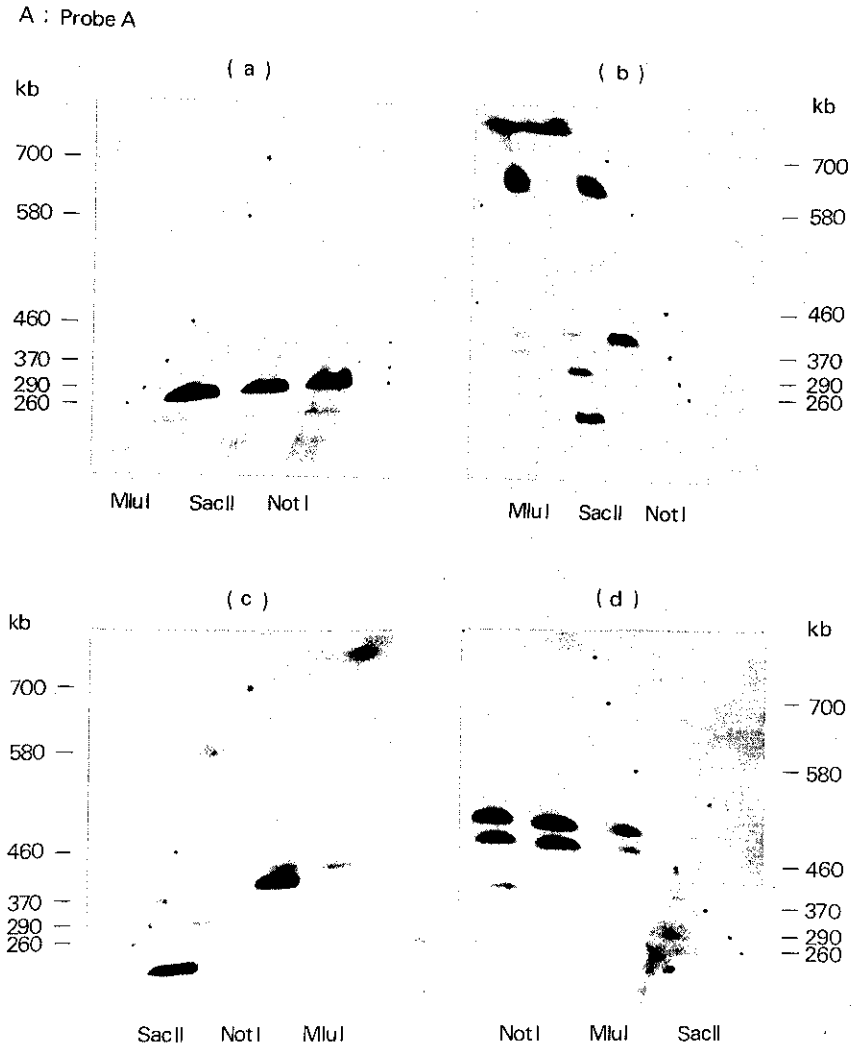
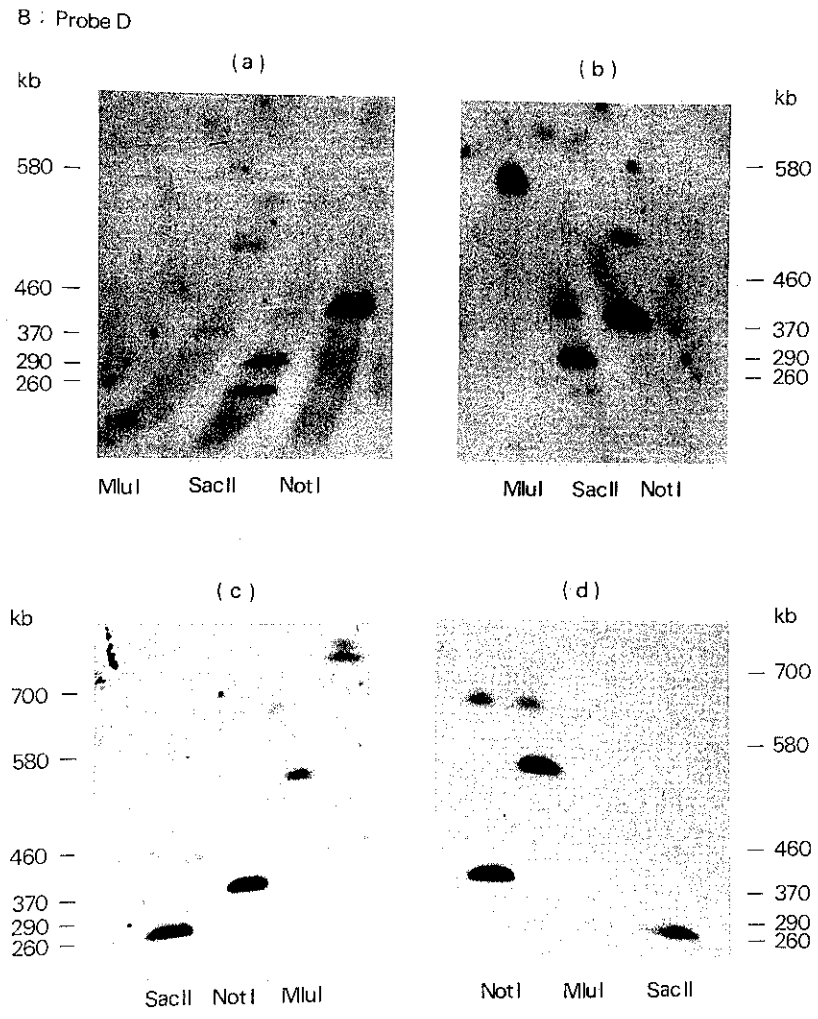


Fig. 4. Southern hybridization analysis of the EGF receptor gene flanking regions in various human tumor cell lines with amplified EGF receptor genes. (A) The 5′-flanking region was analyzed using probe A. Uppermost signals in each lane of (b) were derived from the limiting→

ceptor gene are heterogeneous in A431. The size distribution of 5′- and 3′-flanking regions of the amplified EGF receptor genes in four different tumor cell lines is summarized in Fig. 2D. It is clear that the EGF receptor gene was amplified together with large 5′- and 3′-flanking regions in these carcinoma cells.

Discussion

We have established a mega base scale physical map of the flanking regions of the EGF receptor gene using pulsed-field gel electrophoresis. Utilizing this map information, we have analyzed the structure of the flanking regions of the amplified EGF receptor genes which are commonly found in human squamous carcinoma cells. Among seven cell lines tested, three cell lines carrying EGF receptor genes in more than 20 times the amount in normal cells showed distinct rearrangements within large amplification units.



→ mobility region of the gel above which size separation had not occurred. (B) The 3′-flanking region was analyzed using probe D. a; A431; b, TE8; c, NA; d, HSC2.

The amplified 5′-flanking regions were rearranged in distinct forms unique to each cell line. A431 cells showed 250-kb fragments, the smallest 5′-flanking fragment common to three rare-cutting enzymes, *Mlu*I, *Sac*II and *Not*I. We have already reported that most of the EGF receptor genes amplified in A431 are derived from marker chromosome M4 using A431-mouse A9 cell hybrid, AA1.[26] The 250-kb 5′-fragments were also amplified in AA1 cells, and restriction analysis of the 250-kb fragment revealed a symmetrical structure, suggesting an inverted duplicated structure (unpublished results). So the presence of characteristic fragments common to three rare-cutting enzymes can be explained as a result of formation of inverted duplication. HTF islands of EGF receptor gene, where sites for the three rare-cutting enzymes are clustered, are located in both ends of these characteristic fragments in inverted directions. It is not unreasonable to speculate the

presence of the same inverted duplication in HSC2 and TE8 cells, which also showed characteristic fragments common to the three rare-cutting enzymes. We speculate that the presence of two distinct fragments (480 and 530 kb) in HSC2 cells, corresponding to the 5′-flanking region, may represent two independently derived rearrangement events which result in the formation of inverted duplication. As shown in the physical map of GM130B cells, normal *Sac*II and *Mlu*I sites are located at 290 and 340 kb upstream from the HTF island, respectively. Therefore, if DNA was rearranged between these two sites and formed an inverted duplication, large *Mlu*I and *Not*I fragments should be generated as was seen in TE8 cells. The 630-kb *Sac*II fragment shown as a minor component in this cell line may be an incomplete digestion product, possibly due to partial methylation of *Sac*II sites within the 630-kb fragment and so there may be a *Sac*II recognition site at 630 kb upstream from the HTF island, corresponding to the *Mlu*I and *Not*I sites. The nature of other minor components seen in these cell lines is not clear but they may represent independent amplification events and/or secondary rearrangement events during the amplification process. These three cell lines (A431, HSC2 and TE8) showed characteristic fragments common to the three rare-cutting enzymes, though each set of fragments was different in size and number of amplification units (resulting from each amplification event). Interestingly, cell lines that amplify EGF receptor genes less than 5 times (TE1, HSC 3 and Ca 9-22) did not show any rearrangement under our analytical conditions. They showed the same band pattern as that of GM130B.

The organization of the 3′-flanking region of the amplified EGF receptor gene in three tumor cells (TE8, NA, HSC2) was identical for at least 540 kb away from the HTF island. In A431, however, the 3′-flanking fragment appears heterogeneous, indicating multiple chromosomal rearrangements. One of the major 3′-rearrangement points in A 431 appears between 370 kb (*Not*I site) and 540 kb (*Mlu*I site) downstream of the HTF island because the amplified 370-kb *Not*I fragment but not the amplified *Mlu*I fragment was detected. Size heterogeneity of *Mlu*I fragments indicates that the 3′-flanking regions of the EGF receptor genes amplified in A431 differ from each other depending on their adjacent sequences. Furthermore, additional 3′-rearrangement was observed within the 270-kb *Sac*II fragment, resulting in formation of the 180-kb *Sac*II fragment.

It has been shown that A431 cells produce a 2.9-kilobase aberrant mRNA.[19, 27, 28] A cDNA probe specific for this mRNA, 0.4-kb *Pvu*II-*Cla*I fragment of clone pE15, cannot hybridize with normal EGF receptor mRNA.[19] This unique probe detected 200-kb *Sac*II fragment instead of 270-kb fragment, 540- (*Mlu*I) and 370-kb (*Not*I) fragments in cell lines other than A431 (data not shown). Among them, the *Mlu*I and *Not*I fragments were exactly the same as those detected by probe D. Therefore, the DNA sequence coding for this 3′-portion of aberrant mRNA is located at a position between a *Sac*II site and a *Not*I site downstream of the HTF island (Fig. 2C). We think the production of 2.9-kilobase aberrant mRNA in A431 cells was caused by the rearrangement involving the formation of 180-kb *Sac*II fragment. Thus, in contrast to the well ordered 5′-rearrangement points, the 3′-end rearrangements are distributed in a complex manner in A431 cells, producing both normal and aberrant mRNAs.

It is clear from the present study that amplification events involving the EGF receptor gene appear complex, generating multiple rearrangements. It is tempting to speculate that these rearrangement events may be involved in the mechanisms of inverted duplication. Further studies on the detailed structure of each amplification unit will clarify the mechanisms of EGF receptor gene amplification in human tumor cells.

Recently, c-*erb*B-2 gene,[29] which is also known as *neu*[30] or HER2[31] has been shown to be amplified relatively frequently in human mammary tumors.[32] It is homologous but not identical to c-*erb*B-1 gene encoding EGF receptor. Amplification of the c-*erb*B-2 gene is often accompanied with amplification of the linked c-*erb*A gene,[32] and both genes have been mapped to the q21 region of chromosome 17.[30, 31, 33-35] Interestingly, a transforming retrovirus, avian erythroblastosis virus, has two oncogenes, v-*erb*A and v-*erb*B, derived from host cell genes c-*erb*A and

c-*erb*B, respectively, and mutations in either of these genes lead to loss of some oncogenic properties of this virus, showing that both genes cooperate in cell transformation.[36-38] It is possible that coamplification of genes around the EGF receptor gene may have important roles in the malignant phenotype of the tumors.

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